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Inhibition of *Clostridium botulinum* by *p*-Hydroxybenzoic Acid *n*-Alkyl Esters

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Twelve straight-chain esters, C_5 to C_{14}, C_{16}, and C_{18}, of *p*-hydroxybenzoic acid were prepared, and their melting points, solubilities in water at 25°C, infrared spectra, dissociation constants (pK_a), and activities against *Clostridium botulinum* were determined. These studies also included four commercial straight-chain esters, C_1 to C_4. The most potent activity was exhibited by undecyl and dodecyl esters, which are about 300 times as active as sodium nitrite. Quadratic and cubic equations were developed correlating the activity with pK_a values and chain length of the esters, respectively.

We recently reported the inhibition of *Clostridium botulinum* by 2-*n*-acylamido-5-nitrothiazoles (7). Inhibition was related to the chain length of the acyls, with lauroylamido showing greater activity than the higher or lower homologs.

Short-chain esters of *p*-hydroxybenzoic acid (parabens) have been evaluated for antimicrobial purposes (3, 4, 8-10, 12, 13, 16-18, 21; G. F. D'Alelio, U.S. patent no. 2,269,186, 1942) in food as well as in pure culture. Hirai (8-10) reported activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*. Macias et al. (16) investigated the antimicrobial effect of these parabens against *Salmonella typhi*, *Saccharomyces cerevisiae*, and *Rhizopus nigricans*. Activity was observed to increase with increasing chain length; however, esters beyond *n*-hexyl were not studied. Cavill and Vincent (3) studied the activity of *p*-hydroxybenzoic acid esters against *Aspergillus niger* and *Bacillus fulva*. Murrell and Vincent (18) indicated that these esters inhibited *Aerobacter aerogenes* but found less activity with the higher homologs (*n*-hexyl ester). Robach and Pierson (20) reported that methyl and propyl parabens inhibited toxin production by *C. botulinum* in culture media.

Most bacteria appear to be inhibited more strongly by the higher homologs; however, only compounds with chain lengths to seven carbons have been studied (3, 9, 10, 12, 18, 21). Branched-chain esters possess less activity (3, 16).

Several esters (C_1 to C_8) have been proposed as beer preservatives (F. B. Strandskov and J. B. Bockelman, U.S. patent no. 3,232,766 [to F & M Schaeffer Brewing Co.], 1966) at levels of about 6 μg/ml for heptyl and octyl esters and up to 300 μg/ml for shorter-chain homologs. Methyl, ethyl, and propyl esters have been suggested as substitutes for nitrite in cured meat products for preventing growth of *C. botulinum* (C. N. Sweet, U.S. patent no. 3,899,600 [to Eastman Kodak Co.], 1975).

The study reported here includes methods of preparation, physico-chemical properties, and anticlostridial activity of C_1 to C_{18} straight-chain esters of *p*-hydroxybenzoic acid.

MATERIALS AND METHODS

Preparation of *p*-hydroxybenzoic acid *n*-alkyl esters (C_1 to C_{18}). Methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid were obtained from Sigma Chemical Co., and the *p*-hydroxybenzoic acid and all alcohols were from Aldrich Chemical Co. All commercial materials were about 98% pure and were used without further purification.

Esters of *p*-hydroxybenzoic acid with C_5 to C_{18} alcohols were prepared as follows: 0.1 mol of *p*-hydroxybenzoic acid, 0.5 mol of the corresponding alcohol, and 10 ml of concentrated HCl were placed in a 250-ml, two-necked reaction flask equipped with a condenser and stirrer. The flask was placed in a Wood metal bath at 120 to 140°C for 2 h; during this time about 10 ml of distillate was separated. The temperature was then raised to 160 to 180°C and held for 4 to 6 h. The temperature was lowered to 100°C, the excess alcohol was distilled off in vacuo at 0.1 mm of Hg, and the residue was left overnight at room temperature to solidify. The esters were recrystallized from *n*-hexane, 10 to 20 ml/g; the stearyl ester was recrystallized from acetone, 3 ml/g. Infrared spectra were obtained on all of the compounds. Elemental analyses agreed with theoretical values. Yields of the final products were 85 to 90%.

Determination of water solubility at 25°C. Solubilities of the esters (Table 1) were determined by

placing about 1 g of the compound (weighed to the closest 0.1 mg) into a 100-ml volumetric flask and filling to the mark with distilled water, stirring for 2 h at 25°C, and allowing the suspension to stand overnight at 25°C. The undissolved ester was collected on a preweighed sintered glass funnel of F-porosity and dried in a desiccator over H_2SO_4 at room temperature for 2 days. Solubility was expressed as the difference between added and recovered weights of the samples. Also, the solubilities of C_5 to C_{13} esters were determined spectroscopically. The filtrates were diluted to 3.0×10^{-5} mol/liter, and the solubility was calculated from the absorption maximum at 295 nm; under these conditions maximum solubility was attained in 2 h.

Preparation of the buffers. Buffers of pH 2.00, 7.00, and 10.00 were obtained from Fisher Scientific Co. (no. 50-B-96, 108, and 116, respectively). Buffers of pH 8.50 to 10.50 were prepared from borax solutions, and the remaining buffers were made from dibasic sodium phosphate as outlined by Bates and Bower (1).

***pK*$_a$'s.** Dissociation constants (pK_a's) (Table 1) were determined by the procedure of Danek et al. (5) by use of a Cary model 14 recording spectrophotometer. The esters were dissolved in ethyl alcohol at a concentration of 10^{-3} mol/liter. These solutions were diluted 40-fold with selected buffers, giving final concentrations of 2.5×10^{-5} mol/liter. The spectra of each ester were determined in three different buffers to obtain an absorption maximum at 295 nm and a well-defined isosbestic point or points (14). The spectra of *p*-hydroxybenzoic acid were determined at pH 8.50, 9.20, and 10.85. Isosbestic points appeared at 255 and 227 nm. The pK_a was calculated from the absorption maximum at 280 nm. The spectra of the methyl to nonyl esters were determined in buffers at pH 2.00, 7.00, and 10.00. Isosbestic points appeared at 270 and 230 nm. The pK_a's for all esters were calculated from the absorption maximum at 295 nm. The spectra of decyl, undecyl, and lauroyl esters gave isosbestic points in a buffer system of pH 9.20, 9.50, and 10.85. The isosbestic points appeared at 263 and 235 nm. The spectra of the tridecyl and myristyl esters gave well-defined isosbestic points of 235 and 272 nm in buffers of pH 9.85, 10.85, and 11.45; cetyl and stearyl esters gave points at 250 and 275 nm in systems of pH 10.50, 11.00, and 12.00.

Infrared spectra. A Perkin-Elmer grating spectrophotometer model 421 with KBr disks (1 mg of ester and 220 mg of KBr) was used. Characteristic absorptions at 1,670 and 1,280 cm^{-1} were recorded.

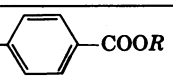
Activity against *C. botulinum* type A. The minimum inhibitory concentration (MIC, in micrograms per milliliter) for *C. botulinum* was determined from a 1- or 10-mg/ml ethanolic solution of the esters by the procedure of Huhtanen (11).

RESULTS AND DISCUSSION

There are several methods for synthesizing *p*-hydroxybenzoic acid esters (6, 9, 12, 13). The procedure described in this paper, with an excess of concentrated HCl used as catalyst, was more practical than these, since the acid could be removed readily by distillation during heating. We found that with increasing chain length of the reacting alcohol, the reaction temperature and time had to be increased. The methyl ester, for example, required 4 h of heating at 100°C; each increase in chain length by $-CH_2-$ required an additional 15 min of heating and an increase by 5°C of the bath. The maximum temperature, however, should not exceed 190°C; otherwise, colored products will be formed.

TABLE 1. *Some physicochemical characteristics of p-hydroxybenzoates*[a]

R	m.p. (°C)	Solubility (g/100 ml of H_2O at 25°C)	pKa	MIC[b] (μg/ml)	Infrared characteristic absorption (cm^{-1})	
					C=O	C—OR
H	213–214	0.4920	9.28	1,000	1,670	1,280
CH_3	127–128	0.2500	8.17	1,000	1,670	1,280
CH_3CH_2	116–117	0.0750	8.22	400	1,670	1,280
$CH_3(CH_2)_2$	96–97	0.0500	8.35	400	1,670	1,280
$CH_3(CH_2)_3$	68–69	0.0170	8.37	200	1,673	1,280
$CH_3(CH_2)_4$	35.5–36	0.0145	8.07	100	1,675	1,280
$CH_3(CH_2)_5$	49–50	0.0379	8.10	20	1,675	1,280
$CH_3(CH_2)_6$	48–48.5	0.1379	8.27	10	1,675	1,280
$CH_3(CH_2)_7$	49–49.5	0.0820	8.44	2.50	1,675	1,280
$CH_3(CH_2)_8$	43–43.5	0.1277	8.80	1.25	1,675	1,270
$CH_3(CH_2)_9$	40–40.5	0.0363	9.39	0.60	1,675	1,275
$CH_3(CH_2)_{10}$	36.5–37.0	0.2368	9.85	0.30	1,678	1,275
$CH_3(CH_2)_{11}$	37–37.5	0.1095	10.46	0.40	1,680	1,275
$CH_3(CH_2)_{12}$	45–45.5	0.0364	11.05	0.60	1,680	1,270
$CH_3(CH_2)_{13}$	56–56.5	0.0364	11.16	1.60	1,685	1,280
$CH_3(CH_2)_{15}$	64–65	0.0379	11.15	10	1,685	1,280
$CH_3(CH_2)_{17}$	58–59	0.0326	11.18	20	1,685	1,280

[a] 

[b] For *C. botulinum*.

The MIC and the physicochemical characteristics determined for these *p*-hydroxybenzoic acid esters are shown in Table 1. The MIC decreased about 3,000-fold with increasing chain length of the ester. Maximum activity, with an MIC of 0.3 to 0.4 μg/ml, was observed with the C_{11} and C_{12} esters. The relationships of ln MIC to chain length, R, and to pK_a were investigated by polynomial regression analysis.

The quadratic equation (equation 1) expresses the significant ($P < 0.01$) relationship between ln MIC and pK_a. A cubic equation (equation 2) describes the significant ($P < 0.01$) relationship between ln MIC and R. This latter equation is shown in Fig. 1.

$$\ln \text{MIC} = 167.74 - 78.14\,pK_a - 9.04\,(PK_a)^2 \quad (1)$$

$$\ln \text{MIC} = 7.29 - 0.32R - 0.1R^2 + 0.006R^3 \quad (2)$$

Melting points of parabens show a gradual decrease, reaching a minimum at the C_5 ester, and then a slight increase.

The spectroscopic method for determining solubility yielded virtually the same results as those obtained from the gravimetric procedure; therefore, only gravimetric results are given (Table 1). The solubilities show a gradual decrease similar to the melting points, reaching a minimum at the C_5 ester, then increase in a zigzag manner. The uneven esters C_7, C_9, and C_{11} are more soluble than the succeeding even esters C_8, C_{10}, and C_{12}, respectively. At C_{13} solubility decreases to 0.0364 g/100 ml and remains almost constant for the higher esters, up to C_{18}. The undecyl ester is the most soluble long-chain paraben (0.2368 g/100 ml).

The pK_a's of the esters were determined spectrophotometrically, since that method was found to be suitable and convenient. The pK_a of *p*-hydroxybenzoic acid was determined to show the validity of our procedure. Our value, pK_a 9.28 (Table 1), correlated well with the conductometric value of 9.32 reported by Kuhn and Wassermann (15).

The infrared spectra of all the *p*-hydroxybenzoic acid esters were essentially identical with the literature spectra of methyl, ethyl, and *n*-propyl *p*-hydroxybenzoates (19), determined in a Nujol mull. Variations were observed in the intensity ratio of the bands at 1,590 and 1,605 cm^{-1}, pertaining to the degenerate isomeric splitting (2). Characteristic absorptions at 1,670 and 1,280 cm^{-1} were recorded, corresponding to the stretching vibrations of C=O and C—OR bonds, respectively (2, 22). With increase of the ester chain the C=O frequencies tend to increase by about 10 units, and those of C—OR tend to decrease by about 5 units (Table 1). The differences in force constants (23) of these shifts correspond to 8,200 and −3,600 dyn/cm, respectively.

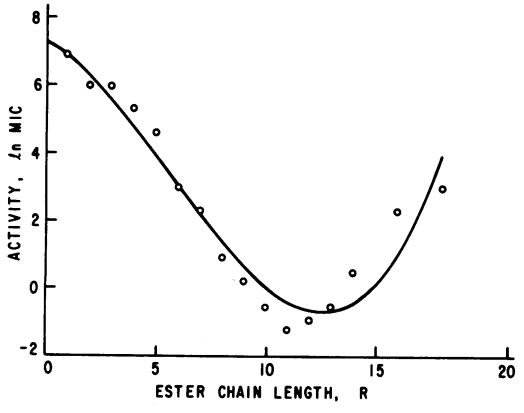


FIG. 1. *Relationship of the chain length (R) of the esters with the minimum inhibitory activity (MIC) against C. botulinum. Circles indicate experimental data; the solid line indicates computerized approximation, described by the cubic equation.*

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